Repurposing toremifene for the treatment of oral bacterial infections

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Running Head: Antibacterial activity and mode of action of toremifene

ABSTRACT

The spread of antibiotic resistance and the challenges associated with antiseptics such as chlorhexidine have necessitated the search for new antibacterial agents against oral bacterial pathogens. As a result of failing traditional approaches, drug repurposing has emerged as a novel paradigm to find new antibacterial agents. In this study, we examined the effect of the FDA-approved anticancer agent toremifene against oral bacteria *Porphyromonas gingivalis* and *Streptococcus mutans.* We found that the drug was able to inhibit growth of both pathogens as well as prevent biofilm formation at concentrations ranging from 12.5 to 25 µM. Moreover, toremifene was shown to eradicate preformed biofilms at concentrations ranging from 25 to 50 µM. In addition, we found that toremifene prevents *P. gingivalis* and *S. mutans* biofilm formation on titanium surfaces. A time-kill study indicated that toremifene acts bactericidal against *S. mutans*. Macromolecular synthesis assays revealed that treatment with toremifene does not cause preferential inhibition of DNA, RNA, or protein synthesis pathways, indicating membrane-damaging activity. Biophysical studies using fluorescent probes and fluorescence microscopy further confirmed the membrane-damaging mode of action. Taken together, our results suggest that the anti-cancer agent toremifene is a suitable candidate for further investigation for the development of new treatment strategies for oral bacterial infections.

KEY WORDS

Toremifene, *Porphyromonas gingivalis*, *Streptococcus mutans*, biofilms, oral infections

INTRODUCTION

Oral infections are among the most common diseases worldwide (1). These infections are typically caused by biofilm-forming bacteria present on the surfaces of both hard and soft tissues (2). The Gram-negative, anaerobic bacterium *Porphyromonas gingivalis* and the Gram-positive bacterium *Streptococcus mutans* are two important causative agents of oral infections. *P. gingivalis* is frequently involved in chronic inflammatory diseases such as periodontitis and peri-implantitis, resulting in the destruction of soft and hard tissues surrounding teeth and dental implants, respectively (3, 4). *S. mutans* is known to be the main pathogenic agent of dental caries, a chronic disease characterized by irreversible destruction of the tooth (5).

Treatment of oral infectious diseases frequently involves the use of anti-infective agents such as chlorhexidine, or in severe cases antibiotics (6, 7). However, the side effects associated with chlorhexidine, such as teeth staining, calculus formation and change of taste sensation, and the development of resistance against antibiotics necessitate the search for alternatives (7, 8). Recently, drug repurposing has gained more attention as an alternative strategy to identify new antimicrobial agents. There are several advantages to repurposing old drugs with known safety and pharmacokinetic profiles over *de novo* drug discovery. Examples include a reduction in time, cost and risks associated with the development of novel antibiotics (9, 10). In an effort to repurpose existing drugs as antibacterial agents, we recently screened the NIH clinical library against *P. gingivalis*. Three compounds were selected that showed potent activity against *P. gingivalis* (toremifene, zafirlukast, and N-arachidonoylaminophenol

(AM404)) (11). The antibacterial activity of toremifene (Figure 1), an FDA-approved drug used in the treatment of breast cancer (12, 13), was further characterized in this study.

The first aim of this study was to assess the antibacterial and antibiofilm activity of toremifene against the oral pathogens *P. gingivalis* and *S. mutans*. Furthermore, the effect of toremifene against oral biofilms formed on titanium, a material frequently used for implant applications, was evaluated. Finally, the antibacterial mode of action of toremifene was investigated. The findings from this study will provide valuable insight into the potential therapeutic application of toremifene for the treatment of oral infectious diseases.

MATERIAL AND METHODS

Bacterial strains and chemicals

P. gingivalis ATCC 33277 was routinely grown on 5 % horse blood agar supplemented with hemin (5 µg/ml) and menadione (1 µg/ml) at 37 °C under anaerobic conditions (90 % N_2, 5 % H_2 and 5 % CO_2) using an Anoxomat AN2OP system (Mart Microbiology, Drachten, the Netherlands). *S. mutans* ATCC 25175 was routinely grown on solid trypticase soy agar (TSB, Becton Dickinson Benelux) containing 1.5 % agar at 37 °C. Liquid cultures of all strains were grown in TSB.

Toremifene was purchased from TCI EUROPE N.V. and stock solutions of 20 mM were prepared in dimethyl sulfoxide (DMSO).

Antibacterial assays

The minimum inhibitory concentration (MIC) of toremifene was evaluated in TSB as described before (14). To determine the minimum bactericidal concentration (MBC), 10 µl aliquots were taken from the wells of the MIC assay that did not show bacterial growth and were plated onto agar plates. After incubation of the plates, the MBC was determined as the lowest concentration of toremifene for which no colony forming units (CFUs) were observed.

Antibiofilm assays

The minimum biofilm inhibitory concentration (MBIC) values of toremifene were determined using crystal violet staining. *P. gingivalis* biofilms were grown anaerobically on the polystyrene pegs of Nunc Immuno-TSP lids (Nunc-Immuno TSP, VWR International) as described previously, with minor modifications (15). Overnight cultures of *P. gingivalis* were diluted 1/10 in TSB. Next, two-fold serial dilutions of toremifene in cell suspension (0 - 200 µM) were prepared at a volume of 150 µl in the polystyrene microtiter plates of the Nunc Device. Subsequently, the plates were covered with a lid containing the pegs and biofilms were allowed to grow on the pegs for 72 h at 37 °C, without shaking. After incubation, the pegs were washed once with phosphate-buffered saline (PBS), stained with 200 µl 0.1 % crystal violet (wt/vol) in an isopropanol-methanol-PBS solution (1/1/18 [vol/vol]) during 1 h and washed with water to remove excess stain and air-dried (0.5 h). Next, the remaining crystal violet stain was removed from the pegs in 200 µl acetic acid (30 %) and the intensity was measured by determining the OD_{570}, using a Synergy MX multimode reader (Biotek, Winooski, VT).

S. mutans biofilms were grown on the bottom of the wells of polystyrene microtiter plates, as they failed to grow on pegs. To this end, overnight cultures were diluted 1/200 in Brain-Heart

Infusion medium (BHI; Becton Dickinson Benelux) supplemented with 3 % sucrose and two-fold serial dilutions (150 µl) of toremifene in the cell suspensions (0 - 200 µM) were prepared in the microtiter plate. After 24 h of biofilm formation at 37 °C, biofilm formation was assessed by crystal violet staining as described above. The lowest concentration of toremifene required to inhibit biofilm formation was defined as the MBIC.

In addition, the biofilm inhibitory effect of toremifene against *S. mutans* and *P. gingivalis* was tested under shaking conditions. Biofilms were grown and quantified as described above, with the difference that biofilms were grown in a shaking incubator.

To determine the effect of toremifene on preformed biofilms, 72 h-old (*P. gingivalis*) or 24 h-old (*S. mutans*) biofilms were grown on polystyrene surfaces as described above. Subsequently, the biofilms were treated with 150 µl growth medium containing toremifene (0 - 200 µM) and were incubated at 37 °C for 24 h. Next, the biofilms were washed with PBS and quantified with cell titre blue (CTB) by adding 200 µl of CTB diluted 1/100 in PBS to each well. After 24 h of incubation in the dark at 37 °C, fluorescence was measured (λ_{ex}: 535 nm and λ_{em}: 590 nm) using the Synergy MX multimode reader (Biotek, Winooski, VT). The minimum biofilm reduction concentration (MBRC) was defined as the lowest concentration of toremifene able to eradicate the preformed biofilm.

Inhibition of biofilm formation on titanium disks

To evaluate the biofilm inhibitory activity of toremifene against *P. gingivalis* and *S. mutans* biofilms grown on titanium, round titanium disks (commercially pure titanium, grade 2; height: 2 mm, width: 0.5 cm) were used. Tests with *P. gingivalis* were performed under anaerobic conditions. First, bacterial suspensions were prepared by diluting overnight cultures

of *P. gingivalis* 1/10 in TSB and of *S. mutans* 1/200 in BHI medium supplemented with 3 % sucrose. Next, the titanium disks were placed at the bottom of the wells of a 96-well plate and were challenged with 200 µl of a bacterial suspension containing 0 to 50 µM toremifene. After 72 h (*P. gingivalis*) or 24 h (*S. mutans*) of incubation at 37 °C under static conditions, disks were removed from the wells and subsequently washed with PBS to remove non-adherent bacteria and placed in centrifuge tubes containing 1 mL PBS. Adherent bacteria were removed from the disks by sonication (45,000 Hz in a water bath sonicator (VWR USC 300-T) for 10 min), followed by vortexing (1 min). Bacterial viability was quantified by serial dilution plating (CFU counts).

In addition, the BacLight LIVE/DEAD bacterial viability staining kit (Molecular Probes, Invitrogen) was used to microscopically evaluate the viability of the biofilms formed on titanium disks. After incubation, the disks were washed with 1x PBS and were transferred to a LIVE/DEAD staining solution containing SYTO 9 and propidium iodide (PI) (prepared according to manufacturer's instructions). After 10 min of incubation at room temperature in the dark, the disks were washed again in 1x PBS and were mounted on a coverslip for imaging. The stained biofilm cells were visualized under a Zeiss Axio imager Z1 fluorescence microscope equipped with a EC Plan-Neofluar 20x objective using the SYTO 9 (λ_{ex} = 483 nm; λ_{em} = 500 nm) and PI (λ_{ex} = 305 nm; λ_{em} = 617 nm) channels.

Time-kill assay

Exponential-phase cells of *S. mutans* were incubated with 1x and 4x the MIC of toremifene or chlorhexidine at 37 °C under shaking conditions (see Table S1). At periodic intervals, aliquots

taken from the samples were serially diluted in $MgSO_4$ and subsequently plated on TSB agar. After incubation for 2 days as 37 °C, cell viability was determined by CFU counting.

Single-step resistance selection

The frequency at which mutants of *P. gingivalis* and *S. mutans* emerge that are resistant to antibacterial agents was determined as described previously (16). Briefly, 500 µl of an overnight culture of *P. gingivalis* or *S. mutans* was plated on agar plates containing antibacterial agents at 5x the MIC (see Table S1). In parallel, the overnight cultures were serially diluted and plated on non-selective agar. After incubation of the plates for 7 (*P. gingivalis*) or 2 (*S. mutans*) days, the MIC of the antibacterial agents for the surviving colonies on selective agar was determined to verify resistance. The spontaneous mutation frequency was calculated by dividing the number of surviving colonies on selective plates by the total number of colonies on non-selective plates after incubation.

Macromolecular synthesis assay

The effect of toremifene on the macromolecular synthesis pathways in *S. mutans* was determined by monitoring the incorporation of radiolabeled precursors of macromolecules. Briefly, *S. mutans* exponential-phase cells (OD_{595} 0.2 to 0.3) were incubated with radiolabeled precursors for DNA ([^{3}H] thymidine (1 µCi)), RNA ([^{3}H] uridine (2.5 µCi)), and proteins ([^{3}H] leucine (2.5 µCi)). Next, the cells were treated with 4x the MIC of toremifene or control antibacterials (ciprofloxacin, rifampicin, tetracycline, triclosan; see Table S1). After 10 min incubation at 37 °C, 100 µl was taken from the samples and was resuspended in 3 ml ice-cold 10 % trichloroacetic acid to stop the reactions and to release free radiolabeled precursors from

cells. Next, the samples were filtered through Whatman 25-mm GF/C glass microfiber filters and were washed three times with 3 ml ice-cold water. Subsequently, the dried filters were transferred to scintillation vials containing 3.5 ml scintillation fluid. Each vial was counted in a liquid scintillation counter (HIDEX 300 SL) for 2 minutes. The results are expressed as percentage of incorporation as compared to the untreated control.

Membrane permeabilization assays

The ability of toremifene to permeabilize the outer membrane of *P. gingivalis* was determined using the fluorescent dye N-phenyl-1-napthylamine (NPN, Sigma, USA) as previously described (16), with some modifications. Briefly, exponential-phase cells were washed and resuspended to an OD_{595} of 0.1 in buffer (5 mM HEPES, pH 7.4). Next, toremifene (0 - 25 µM) and NPN (10 µM) were added and changes in fluorescence were recorded after incubation for 5 minutes using a Synergy MX multimode reader (Biotek, Winooski, VT) (λ_{ex}: 350 nm and λ_{em}: 420 nm). Triclosan at 1x the MIC was used as a positive control because of its strong outer membrane permeabilizing properties. Ciprofloxacin at 1x the MIC was used as a negative control (see Table S1).

The ability of toremifene to permeabilize the inner membrane of *P. gingivalis* and the membrane of *S. mutans* was determined using the fluorescent dye SYTOX green (Invitrogen, USA) as previously described (16). Briefly, exponential-phase cells were washed and resuspended to an OD_{595} of 0.5 in PBS. Next, cells were incubated with toremifene (0 - 25 µM) and SYTOX green (1 µM) at 37 °C for 15 min. Thereafter, the increase in fluorescence was measured using a Synergy MX multimode reader (Biotek, Winooski, VT) (λ_{ex}: 504 nm and λ_{em}: 523 nm). Melittin (10 µg/ml for *P. gingivalis*; 2.5 µg/ml *S. mutans*) was used as a

positive control because of its strong inner membrane permeabilizing properties. Ciprofloxacin at 1x the MIC was used as a negative control (see Table S1). The fluorescent values of each condition were divided by the respective OD_{595} values to correct for the cell density of the culture. In addition, this ratio was corrected for background fluorescence by subtracting fluorescent values of untreated cells.

BODIPY-TR-cadaverine displacement assay

To determine the ability of toremifene to bind with the lipid A part of lipopolysaccharides (LPS), the fluorescent probe Bodipy TR cadaverine (BC) (Thermo Fisher Scientific, USA) was used. In this study, exponential-phase cells of *P. gingivalis* were washed and resuspended to an OD_{595} of 0.3 in PBS. Next, cell suspensions were transferred to the wells of a black 96-well microtiter plate and were mixed with 2.5 µM Bodipy TR cadaverine. After 2 h of incubation at 37 °C, a 2-fold serial dilution of toremifene (0 - 50 µM) was added to the wells. Then, fluorescence was assessed for 30 min using a Synergy MX multimode reader (Biotek, Winooski, VT) (λ_{ex}: 580 nm and λ_{em}: 620 nm). Cells treated with 1x the MIC of ciprofloxacin were used as a negative control (see Table S1). Cells treated with 1x and 4x the MIC of chlorhexidine were used as a positive control (see Table S1). BC displacement from LPS was calculated using the formula $((F - F_0)/(F_{max} - F_0)) \times 100$, where F_{max} is the fluorescence intensity of BC without cells, F_0 is the intensity in the presence of cells alone, and F is the intensity of the mixture of cells and BC at varying concentrations of toremifene, chlorhexidine or ciprofloxacin.

Fluorescence microscopy

Exponential phase cells of *P. gingivalis* and *S. mutans* were treated with 4x the MIC of toremifene or control antibacterials (ciprofloxacin, rifampicin, tetracycline, triclosan; see Table S1). After 30 min of incubation at 37 °C, cells were centrifuged and stained with 10 µg/ml N-(3-triethylammoniumpropyl)-4-(p-diethylaminophenyl-hexatrienyl) pyridinium dibromide (FM 4-64, Molecular Probes) for 10 min at room temperature before being imaged. Cells were visualized using a Zeiss Axio imager Z1 fluorescence microscope equipped with a EC Plan-Neofluar 100x objective, using the FM 4-64 channel (λ_{ex} = 506 nm; λ_{em} = 751 nm).

Hemolysis assay

The test was performed as described previously, with some modifications (17). Briefly, fresh horse red blood cells (RBCs) were rinsed three times with PBS by centrifugation for 10 min at 800 g and diluted in PBS to achieve a final RBC concentration of 4 %. The resulting suspension was incubated at 37 °C for 10 min under shaking conditions. Subsequently, 200 µl of the suspensions were transferred to the wells of a microtiter plate and the assay was initiated by addition of different concentrations of toremifene to the suspensions. Controls included RBC suspensions treated with PBS and with triton X-100 (1 %) to provide reference for 0 % and 100 % hemolysis, respectively. The resulting suspensions were incubated for 60 min at 37 °C. Following centrifugation for 10 min at 800 g, hemolysis was assessed by measuring the absorbance of the supernatant at 540 nm. Percentage of hemolysis was calculated relative to 100% hemolysis with Triton X-100.

Cytotoxicity assay

A cytotoxicity test of toremifene was performed on a cell type relevant to the oral cavity homeostasis, with the aim of screening for concentrations that do not inhibit cell growth or induce cell death. HOC18 cells, an immortalized human oral gingival epithelial cell line, were used (18). Cells were plated in 96-well plates at 15000 cells/well in Minimum Essential Medium Eagle-Alpha Modification (αMEM; Sigma, Bornem, Belgium) with 0.292 g/l l-glutamine (G7513; Sigma, Bornem, Belgium) supplemented with 10 % fetal bovine serum (PAA Laboratories GmbH, Pasching, Austria) and 1 % antibiotic-antimycotic (Gibco® 15240, Life Technologies SAS, Saint Aubin, France). Cells were maintained overnight at 37 °C in a humidified environment with 5 % CO_2.

At day 1 post-seeding, cells were incubated with toremifene by adding the compound to the culture medium. A two-fold serial dilution assay of toremifene was used, starting from 50 µM. Suspensions of the same cell line under the same conditions exposed to triton X-100 (5 %) or cultured without chemicals were used as controls. The proliferation of the HOC18 cells in the presence or absence of chemicals was investigated after 1 day of compound addition. Cell viability was monitored using the XTT assay according to manufacturer instructions (XTT Cell proliferation Kit II, Roche Diagnostics GmbH, Roche Applied Science, Penzberg, Germany). Briefly, this is a colorimetric assay, performed by adding XTT solution 4 h prior the end of toremifene (or triton X-100) exposure. In this assay, metabolically active cells cleave the yellow tetrazolium salt to form the orange formazan dye, whose absorbance is recorded at 450 nm and 650 nm (reference wavelength), using a spectrophotometer (Multiskan Ascent 96/384, Thermo Scientific, Waltham, MA, USA), associated with Ascent software version 2.6 (Thermo Electron Corporation, P.O. Box 100 FIN-01621, Vantaa,

Finland). Percentage of cytotoxicity was calculated relative to 100% cytotoxicity with Triton X-100.

Statistical analysis and reproducibility of the results

Statistical analysis was performed by one-way ANOVA, followed by Dunnett's multiple comparison test. p-values < 0.001, < 0.01, and < 0.05 were considered to be statistically significant. All experiments were repeated at least three times.

RESULTS

Antibacterial and antibiofilm activity of toremifene against *P. gingivalis and S. mutans*

In a previous screening of a drug-repositioning library, the anticancer drug toremifene was identified as a new antibacterial compound that shows activity against *P. gingivalis* (11). To further evaluate the antibacterial potential of toremifene against oral bacteria, its activity was investigated against the prominent oral pathogens *P. gingivalis* and *S. mutans* using MIC, MBC, MBIC, and MBRC assays. Strikingly, as evidenced from Table 1, the activity of toremifene against planktonic and biofilm cultures is similar, underlining the antibacterial potential of this compound. In addition, we find that toremifene is active against biofilms grown under shaking conditions.

Activity of toremifene against *P. gingivalis* and *S. mutans* biofilms grown on titanium disks

Titanium has a high level of biocompatibility, making it a very suitable implant material for dental implants (19). Therefore we tested if toremifene remains active against *P. gingivalis* and *S. mutans* biofilms grown on titanium disks. As shown in Figure 2A and B, toremifene concentrations of 25 and 12.5 µM significantly reduce biofilm formation on titanium disks by *P. gingivalis* and *S. mutans*, respectively. In addition, the LIVE/DEAD bacterial viability kit was used to visualize the viability of biofilms formed on titanium surfaces (Figure 2C). This kit contains two dyes: SYTO 9, which stains live bacteria green and PI, which stains bacteria with compromised membranes. Compared to the untreated disks, a lower number of viable green cells is detected on the titanium disks incubated from a concentration of 25 µM toremifene for *P. gingivalis* and from a concentration of 12.5 µM toremifene for S. *mutans*, thereby corroborating the results of the CFU counts.

Time-kill assay of toremifene and chlorhexidine against *S. mutans*

To investigate the bactericidal activity of toremifene, and to compare it with the activity of the commonly used antiseptic chlorhexidine, time-kill assays were performed (Figure 3). For a number of practical reasons (e.g. sampling at different time points under anaerobic conditions), we decided to assess the killing kinetics of toremifene against *S. mutans* instead of *P. gingivalis*. A clear bactericidal effect is observed when *S. mutans* cells are incubated for 24 h at 1x and 4x the MIC of toremifene, as can be seen by a reduction in cell counts by 6.2 $\log_{10}$ CFU/ml and 6.5 $\log_{10}$ CFU/ml, respectively. Chlorhexidine exhibits a much slower bactericidal activity, with only a reduction in cell counts by 4.8 $\log_{10}$ CFU/ml after 24 h incubation with 4x the MIC. Regrowth is observed after 24 h incubation with 1x MIC of chlorhexidine.

Single-step resistance selection

For an antibacterial agent to remain effective during treatment, emergence of resistance should be minimal. For this reason, we attempted to determine the frequency at which mutants resistant to toremifene appear. However, no spontaneous toremifene-resistant mutants of *S. mutans* could be generated (mutation frequency < 4.7×10^{-9}). In contrast, rifampicin-resistant mutants of *S. mutans* were obtained with an average mutation frequency of $1.24 \pm 0.9 \times 10^{-8}$. Similarly, no spontaneous toremifene-resistant mutants of *P. gingivalis* could be recovered (mutation frequency < 5.93×10^{-9}).

Effect of toremifene on macromolecular synthesis pathways

The effect of toremifene on three macromolecular synthesis pathways (DNA, RNA, and protein synthesis) was tested by determining the incorporation of radiolabeled precursors into macromolecules after short exposure of *S. mutans* to 4x the MIC of toremifene (Figure 4). The effect of toremifene on a specific macromolecular synthesis pathway was compared to the effect after treatment with 4x the MIC of a known inhibitor of this pathway (ciprofloxacin (DNA synthesis), rifampicin (RNA synthesis), and tetracycline (protein synthesis)). In addition, negative controls were included in all assays (tetracycline for DNA and RNA synthesis, ciprofloxacin for protein synthesis). Treatment with toremifene causes a moderate inhibition of incorporation of precursors into all tested macromolecules and does not result in a preferential inhibition. These results are typical for treatment of bacterial cells with a membrane-damaging agent (20–23). Indeed, treatment of the cells with the membrane-

damaging antibacterial agent triclosan causes a similar effect as toremifene on the incorporation of precursors into macromolecules.

Effect of toremifene on membrane permeability

To investigate the membrane-damaging effects of toremifene on the outer membrane of *P. gingivalis*, the hydrophobic fluorescent probe NPN was used. Normally, NPN cannot partition into the membrane due to the presence of lipopolysaccharides. However, when the outer membrane is damaged, NPN can enter the phospholipid layer, which results in increased fluorescence (24). As shown in Figure 5A, treatment of the bacteria with increasing concentrations of toremifene results in an increased uptake of NPN in the membrane. These results indicate that toremifene alters outer membrane permeability. To determine the effect of toremifene on the inner membrane of *P. gingivalis* and the membrane of *S. mutans*, the nucleic acid stain SYTOX green was used. This stain does not penetrate the inner membrane of bacteria. However, when the inner membrane is permeabilized, SYTOX green can enter the cell and bind to nucleic acids, thereby emitting a strong fluorescent signal (25). As seen in Figure 5B and 5C, SYTOX green uptake is increased with increasing concentrations of toremifene, indicating that the compound is also capable of permeabilizing the inner membrane of *P. gingivalis* and the membrane of *S. mutans*.

Binding of toremifene with LPS

Next, we examined the interaction between toremifene and LPS of *P. gingivalis*, using the Bodipy TR cadaverine displacement assay. Bodipy TR cadaverine is a fluorescent probe that strongly binds to the lipid A moiety of LPS. When a compound is added that interacts with

LPS, Bodipy TR cadaverine is displaced from the complex, which results in increased fluorescence (26). A fast increase in fluorescent signal is observed after treatment with different concentrations of toremifene, suggesting that the compound binds with high affinity (Figure 6).

Microscopic visualization of membrane damage

To further examine the effect of toremifene on the membrane, fluorescence microscopy was employed using the membrane stain FM 4-64. Treatment of the cells with solvent control (DMSO) results in intact homogeneously stained membranes (Figure 7). On the other hand, treatment of the cells with 4x the MIC of toremifene, results in disrupted membranes (Figure 7). Furthermore, the latter observations are comparable to those obtained after treatment of the cells with 4x the MIC of triclosan. This phenotype is not observed after treatment of cells with antibiotics with different modes of action (ciprofloxacin, rifampicin, and tetracycline (Figure S1)).

Hemolytic activity and cytotoxicity

Repurposing of existing drugs offers the advantage of known safety and pharmacokinetic profiles. However, for novel applications, cytotoxicity of these compounds remains to be investigated. We assessed the hemolytic activity of toremifene against horse red blood cells as well as its potential cytotoxic effect on a human oral gingival epithelial cell line (HOC18). As shown in Figure 8A, concentrations of toremifene as high as 100 µM do not cause hemolysis, indicative of good hemo-compatibility. Conversely, exposure for 24 h to toremifene concentrations exceeding 25 µM is toxic to HOC18 cells (Figure 8B).

DISCUSSION

Known side effects of currently used antiseptics and the rising threat of antibiotic resistance demonstrate the need for the development of novel therapies to treat oral infections. In an attempt to identify new drugs with potent activity against the oral pathogen *P. gingivalis*, we recently performed a screen of a repurposing library (11). From this screening, toremifene was withheld for further characterization. Toremifene is an FDA-approved anticancer drug used in the treatment of breast cancer (12, 13). This compound is known to bind with the estrogen receptor, thereby interfering with the estrogen-mediated growth stimuli of tumor cells (13). Earlier studies have already reported the potency of toremifene in other applications. As such, toremifene has been reported to have antibacterial activity against *Francisella novicida*, a model organism of the tularemia-causing pathogen *Francisella tularensis*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* (27, 28). Furthermore, we and others have reported on the antifungal effects of toremifene (28–30). In addition, toremifene has antiviral activity against Ebola viruses (31). However, to our knowledge, no data exist on its activity and mode of action against oral bacterial pathogens. Likewise, no extensive study exists on the antibacterial mode of action of toremifene.

We report here that toremifene displays potent activity against the prominent oral pathogens *P. gingivalis* and *S. mutans,* making it a potential candidate for use as a new antibacterial agent. Of note, studying bacterial killing kinetics revealed fast killing by toremifene as compared to the antiseptic chlorhexidine, which is likely to have a positive effect on treatment

outcome. Furthermore, we found that toremifene has a low tendency for selection of spontaneous resistant mutants, adding to its potential as a novel therapeutic.

Understanding the mode of action of toremifene is crucial for its development as a potential antibacterial agent. To get a first idea about its mode of action, a macromolecular synthesis assay was conducted. We were unable to perform this assay under strict anaerobic conditions necessary to avoid physiological changes caused by oxidative stresses in *P. gingivalis* cells (32). Therefore, we performed the assay using *S. mutans* cells for which we found that toremifene moderately inhibits the synthesis of all tested macromolecules. These data suggest that toremifene possibly acts by disrupting the integrity of the bacterial membrane (20–23). Subsequently, we validated that toremifene rapidly permeabilizes the outer and inner membrane of *P. gingivalis* and the membrane of *S. mutans*. In addition, we showed that toremifene is able to interact with the LPS of the outer membrane of *P. gingivalis*, which further confirms a direct interaction of toremifene with bacterial membranes. Finally, we microscopically visualized the changes in bacterial membrane integrity. Non-homogeneously stained membranes were observed after treatment of both *P. gingivalis* and *S. mutans* with toremifene. Combined, these results indicate that membrane damage likely is the primary antibacterial mode of action of toremifene, which is in accordance with previous studies. Indeed, Dean & van Hoek (2015) demonstrated that toremifene at a concentration of 5 µM strongly permeabilizes the membrane of the Gram-negative bacteria *F. novicida*. Furthermore, Delattin *et al.* (2014) found that toremifene at a concentration of 12.5 µM induces membrane permeabilization in *C. albicans* biofilm cells. However, further work is needed to identify the molecular mechanisms behind the observed membrane damage.

Thanks to their potentially rapid bactericidal effects, activity against both growing and dormant populations and low potential for resistance development, membrane-acting agents are believed to be good candidates for treating biofilm-related persistent infections (33). Recently, the activity of toremifene against *S. aureus* biofilms formed under *in vivo* conditions has been described, thereby further highlighting the potential of this compound to be used in treatment of biofilm-related bacterial infections (28). However, to evaluate the potential of toremifene for application against oral infections, additional experiments should be conducted using a relevant *in vivo* model (34). In addition, special attention should be paid to the fact that in nature, biofilms often exist of multiple bacterial species, underscoring the need for investigating the activity of toremifene against mixed-species biofilms formed on different surfaces (35).

Usually, in treatment of breast cancer, patients receive toremifene orally at a dose of 60 mg/day. Some clinical studies even mention the use of toremifene at a dose of 680 mg/day, which lies well in the range of recommended antibiotic dosages for treatment of oral infections (13, 36). Regarding toxicity, toremifene is generally well-tolerated by patients (13). Most common side effects include hot flushes, sweating, nausea and vaginal discharge, and serious adverse events are rare (13). This is in accordance with our data showing good hemo-compatibility and limited cytotoxicity. It should be noted that, compared the toxicity assay conditions, shorter treatments (e.g. in the case of mouthwashes) are likely to be even less detrimental. These findings further pave the way to repurpose the compound for antibacterial therapeutic uses.

In conclusion, we demonstrated that the anticancer drug toremifene displays antibacterial activity against planktonic and biofilm cells of the prominent oral bacterial pathogens *P.*

457 *gingivalis* and *S. mutans*. Moreover, we showed that toremifene effectively kills these bacteria
458 in a rapid manner by damaging the bacterial membrane. Future experiments including *in vivo*
459 studies will be necessary to fully reveal the potential of toremifene to be used in the treatment
460 of oral bacterial infections.
461
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468
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Commented [GE1]: Wat hiermee doen?

Commented [NV2R1]: Under review van maken? Het is nog wat vroeg om te informeren naar de status vrees ik?

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573

574 **FIGURE LEDGENDS**

575

576 **Figure 1.** Structure of toremifene (pKa 8.0).

577

578 **Figure 2.** Reduction of *P. gingivalis* (A) and *S. mutans* (B) biofilm formation on titanium
579 disks by toremifene. Percentage of biofilm formation in the presence of toremifene relative to
580 the untreated control. Values are means ± SD of three independent experiments. *p < 0.05,
581 **p < 0.01, ***p < 0.001 as compared with the untreated control. (C) Fluorescence
582 microscopy images of biofilms formed on titanium disks. Live cells are stained green, cells
583 with compromised membranes are stained red. Images were processed with unsharp mask of
584 Zen 2.0. The scale bars represent 100 µm.

585

586 **Figure 3.** Time-kill kinetics of toremifene against *S. mutans*. (A) Exponential-phase cells of
587 *S. mutans* were treated with 1x the MIC and 4x the MIC of toremifene (TOR), with 1x the
588 MIC and 4x the MIC of chlorhexidine (CHX) or with the solvents of the drugs (DMSO and
589 water, respectively). Samples were taken at 0, 1, 2, 3, 4, 5 and 24 h and CFUs/ml were
590 determined. All data represent means ± SD from 3 independent experiments. The dotted line
591 indicates the lower limit of detection.

592

593 **Figure 4.** Percentage of incorporation of radiolabeled precursors into macromolecules after
594 treatment of *S. mutans* with 4x the MIC of toremifene (TOR) or control antibacterials
595 (ciprofloxacin (CIP), rifampicin (RIF), tetracycline (TET), triclosan (TRI)). Data represent the
596 means of at least three independent replicates ± SD.

Commented [NV3]: Hier is tor gearceerd, in volgende figuren is tor telkens zwart terwijl de controles gearceerd of wit zijn
Uniform maken?

Figure 5. Effect of toremifene on membrane permeability. (A) Outer membrane permeabilization of *P. gingivalis* after treatment with different concentrations of toremifene, assessed by quantifying NPN uptake. Cells treated with 1x the MIC of triclosan (TRI) were used as a positive control (see Table S1). (B) Inner membrane permeabilization of *P. gingivalis* after treatment with different concentrations of toremifene, determined by measuring SYTOX green uptake. Melittin (MEL) (10 µg/ml) was used as a positive control. (C) Effect of increasing concentrations of toremifene on the membrane permeability of *S. mutans*, monitored by the uptake of SYTOX green. Cells treated with melittin (MEL) (2.5 µg/ml) served as a positive control. For both (A), (B), and (C), cells treated with ciprofloxacin (1x the MIC) served as a negative control. Data represent the means of three independent replicates ± SD (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Figure 6. Determination of the binding affinity of toremifene for LPS of *P. gingivalis* using Bodipy TR cadaverine (BC). The concentration-dependent displacement of Bodipy TR cadaverine from LPS induced by toremifene is shown. Cells treated with 1x and 4x the MIC of chlorhexidine (CHX) were used as a positive control (see Table S1). Cells treated with 1x the MIC of ciprofloxacin (CIP) were used as a negative control (see Table S1). Data represent the means of three independent replicates ± SD.

Figure 7. Microscopic visualization of toremifene-induced membrane damage using the lipophilic dye FM4-64. Cells were either treated with DMSO (solvent control) or with 4x the

MIC of toremifene (TOR) or triclosan (TRI). Scale bars correspond to 2 µm. Images were processed with unsharp mask of Zen 2.0.

Figure 8. Effect of toremifene on mammalian cells. (A) Dose-response of the hemolytic activity of toremifene towards red blood cells. Red blood cells were treated with different concentrations of toremifene, and its hemolytic activity was determined in comparison with Triton X-100 (100 % hemolysis) and PBS (0 % hemolysis). Tests were performed in quadruplicate, and the results are presented as means ± SD. (B) Dose-response of the cytotoxic activity of toremifene towards HOC18 cells. Cytotoxicity was determined in comparison with Triton X-100 (positive control) and supplemented αMEM medium (0 % cytotoxicity). Tests were performed in duplicate, and the results are presented as means ± SD.

Commented [NV4]: Panel B: moet Y as tot 150 lopen? Dat geeft boven een grote witruimte?

Niet zeker of triton en negatieve controle op deze figuur horen, daarnaar is er genormaliseerd, geeft beetje artificiële look, nee?

TABLES

Table 1. MIC, MBC, MBIC, MBRC values of toremifene against oral pathogens[1]

	MIC (µM)	MBC (µM)	MBIC (µM)	MBRC (µM)
P. gingivalis	12.5	25	12.5 / 12.5*	25
S. mutans	25	50	25 / 12.5*	50

[1]Abbreviations: MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; MBIC, minimum biofilm inhibitory concentration; MBRC, minimum biofilm reduction concentration

* MBIC values determined under shaking conditions